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Subject: Fish trend publications to consider
Date: Friday, April 26, 2013 10:39:49 AM
Attachments: [Stow-MixedOrderModel.pdf](#)
[Hebert and Keenleyside-lipid normalizing.pdf](#)
[Area1_SedimentCollectedDate.xlsx](#)

Hello Folks,

As promised yesterday, please find attached: 1) the Stow publication I promised to forward yesterday, 2) the Area 1 sediment data I used for SWAC analysis, as well as 3) the Hebert and Keenleyside paper supporting part of the rationale for using lipid and length as co-variates in the regression models rather than dividing PCB concentrations by lipid prior to analysis. For any questions, I'll be available today on my cell phone at 509-432-6400

Some notes follow:

SWAC:

The attached spreadsheet includes key columns used to subset the data highlighted in yellow. These columns identify biased vs unbiased sampling designs as well as removed sediments and finally those samples that are analytical duplicates.

Samples satisfying these if statements were included in the analysis:

```
if Top_in eq 0;  
if param = "Total_PCB";  
if Removed eq "NO";  
if SampleType ne "DUP";  
if SampleDesign eq "Unbiased";  
if missing(ConvResult) eq 0;
```

And to select specific groups of years for inclusion in the analysis--the following is used to get year collected, rather than the year of laboratory analysis which sometimes differ greatly.

```
if YearCollected eq 1993 or YearCollected eq 2000 or YearCollected eq 2007;
```

Treatment of Lipid in Trend Analysis:

The Hebert paper illustrates that the regression approach provides a more flexible model relaxing the assumptions of 1) linearity between lipid and PCB, and 2) regression through the origin, and 3) allows full understanding of the relationship between lipid and PCB which is assumed in the lipid normalized approach.

For estimating exponential decay rates, either normalization or treating lipid as a predictor in the regression model will usually result in similar estimates of the decay rate, but the lipid normalization approach fails to capture components of uncertainty needed for projecting whole-tissue PCB trends into the future. The whole tissue trends are needed to compare future

concentrations with risk-based or other administrative thresholds.

For extrapolating whole-tissue PCB concentrations into the future, only the regression approach provides a means to estimate uncertainty in these forward predictions. Importantly, the confidence and prediction intervals are functions of the covariance between trend model parameters (time, lipid, length). Because the time to specified threshold values is central to some RAO's, forward projections of concentration are important to decision makers, and their uncertainty bounds are needed to provide an understanding of the reliability of projections of time required to attain threshold values.

One additional item that came up on the call yesterday. There was discussion of elimination of fish that were below the legal size limits for sport capture and consumption. I would suggest weighing this approach against using these samples in the analysis and adjusting for the effects of length through the regression. Inclusion of these samples should improve the precision of estimates and would only be problematic if the length to PCB relationship falls apart for smaller fish. I would suggest looking at that question when evaluating the use of these smaller fish. I used them, but did not do a careful analysis to determine if there were any negative affects on the quality of the regression models.

The regression approach is described in more detail in sections 4.2.1-4.2.3 in the February 13 report available [here](#).

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A MIXED-ORDER MODEL TO ASSESS CONTAMINANT DECLINES

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Abstract. We introduce a generalized form of the common first-order (exponential) decay model, that has potential utility for describing contaminant declines in environmental applications, particularly when declines are a mixture of many underlying processes. The exponent on contaminant concentration is left as a free parameter allowing the order of the reaction to be determined by the data. The mixed-order model is more flexible than models with the exponent determined a priori, facilitating an improved fit to observed behavior. We demonstrate the utility of this model, and compare it to two other models, by estimating PCB concentration declines in Lake Michigan fishes.

Keywords: BOD, mixed-order model

1. Introduction

It is common in environmental modeling to assume processes occur at rates proportional to the concentration of the substance of interest. Specifically, many environmental processes are described mathematically as:

$$\frac{dC}{dt} = -kC \quad (1)$$

where C is a concentration (mass/volume), t is time and k is a rate constant (1/time). The integrated form of this expression is the familiar first-order or exponential decay formula:

$$C_t = C_0 e^{-kt} \quad (2)$$

where C_t is the concentration of C at time t , C_0 is the initial concentration of C , and e is the natural logarithm base. Environmental processes that have been approximated this way include biochemical oxygen demand (BOD) decay (Tchobanoglou and Schroeder, 1985) and the decline of organochlorine pollutants in the Great Lakes (Devault *et al.*, 1986). This approach has intuitive appeal, and the parameters C_0 and k are easy to estimate using simple linear regression, given only a few measurements of C_t .



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The process in Equation (1) is referred to as a ‘first-order’ reaction. The term first-order arises from an implicit exponent of ‘1’ on the quantity C :

$$\frac{dC}{dt} = -kC^1 \quad (3)$$

The assumption of a ‘1’ in the exponent is so common and subtle that many researchers scarcely recognize a subjective decision has been made by choosing a first-order model (Berger and Berry, 1988). For fundamental processes such as chemical reactions and nuclear decay, the first-order assumption has sound theoretical or empirical support. However, environmental processes occur at a very different scale than chemical reactions and are an aggregation of numerous underlying, possibly unknown, individual processes. The first-order assumption is useful when the pollutant is a passive substance in a particular medium. The rate constant then refers to the turnover rate of the medium in a locally defined region. However, when chains of multi-compartment processes are represented in a simple equation the rate parameter actually represents a complex mixture of dynamics. An alternative to fixing the exponent in Equation (3) to a value of one is to leave the exponent as a free parameter to be estimated from the observations. Equation (3) is more generally expressed as:

$$\frac{dC}{dt} = -kC^\theta \quad (4)$$

with the integrated form:

$$C_t = \left\{ C_0^{1-\theta} - kt(1-\theta) \right\}^{\frac{1}{1-\theta}} \quad (5)$$

where θ is a pseudo-order parameter (POP), and Equation (5) is a mixed-order model (MOM). When $\theta = 0$ concentration decreases linearly to zero (Figure 1). When θ is greater than 0 the rate of concentration decrease slows with time, with a zero asymptote. When $\theta = 1$, Equation (4) becomes (by definition) Equation (2), and the rate of decrease is proportional to C ; in other words the rate of decrease goes down with time. Larger θ s indicate faster initial concentration decreases, followed by increasingly slower decreases. For given k values, sufficiently large θ s result in concentration decreases that, beyond a certain time, are almost undetectable unless a relatively long time interval is observed. θ is not restricted to integers, fractional values are also possible.

2. Application to Lake Michigan PCB Data

The mixed-order model in Equation (5) has application for tracking PCB concentration decreases that have occurred in biota since US PCB production was banned

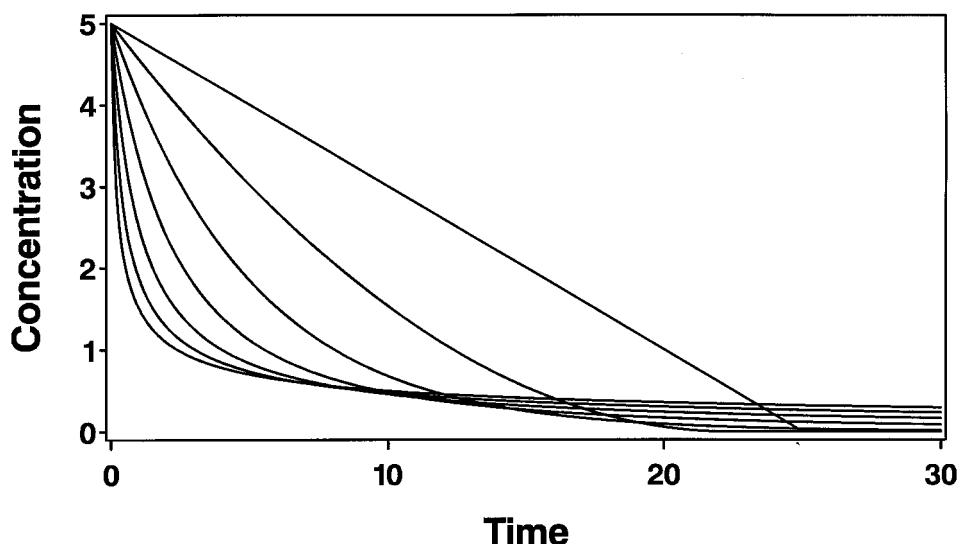


Figure 1. Examples of the mixed-order model with θ ranging from 0 (top, straight line) to 3.0 in 0.5 increments for given C_0 and k .

and usage curtailed in the 1970s. Early reports following restrictions indicated fish PCB concentrations appeared to reflect first-order declines (Devault, 1986). As time passed, though, the rate of decline appeared to slow more than expected under first-order dynamics (Federal Register, 1993). Stow *et al.* (1995) compared the fit of a first-order model with two alternatives: a first-order model with a non-zero asymptote (C_a):

$$C_t = C_0 e^{-kt} + C_a \quad (6)$$

and a double exponential model (the sum of two first-order processes), and found both alternatives fit the data better than did the first-order model, with the non-zero asymptote model generally describing overall system dynamics best. The non-zero asymptote model has also been used to examine PCB concentration declines in Great Lakes herring gull eggs (Stow, 1995) and to explore deterministic factors influencing herring gull egg PCB concentration declines (Hebert *et al.*, 1997).

However the non-zero asymptote model restricts PCB concentrations to remain above an estimated asymptote, C_a ; perhaps an unrealistic long-run constraint. The mixed-order model remedies this limitation, by allowing initial rapid declines, followed by very slow secondary declines, dynamics similar to those observed in Great Lakes biota PCB levels.

3. Methods

Data were provided by the Wisconsin and Michigan Departments of Natural Resources. Sampling and analysis protocol have been previously detailed (Stow *et al.*, 1995). The fish included in this analysis are of varying size and age and have been collected from locations, within Lake Michigan, that have varied over time. Analyses were conducted using skin-on filets reflecting the portion of fish consumed by humans.

We estimated parameters for the first-order model (Equation (2)), the non-zero asymptote model (Equation (6)) and the mixed-order model (Equation (5)) for PCB concentrations from five species of Lake Michigan fish: brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), lake trout (*Salvelinus namaycush*), and rainbow trout (*Oncorhynchus mykiss*). Parameters were estimated under a log transformation of both sides of each equation to achieve equal variance of the disturbance term over time. We estimated model parameters using linear least squares for Equation (2) and nonlinear least squares for Equations (5) and (6). The nonlinear models were fit using the Gauss-Newton algorithm as implemented in SAS software. Composite samples (multiple fish chemically analyzed as a single sample) were weighted by the number of fish in the sample. For all species except brown trout, 1974 was considered year zero. 1978 was year zero for brown trout.

To compare the relative fit to data of the first-order, mixed-order, and non-zero asymptote models we used the extra sum-of-squares criterion (Bates and Watts, 1988):

$$\frac{(ess_r - ess_u)/(df_r - df_u)}{mse_u} \sim F_{(df_r - df_u)}, df_u$$

where ess_r and ess_u are the restricted and unrestricted error sums of squares, respectively, df_r and df_u are the restricted and unrestricted error degrees of freedom, respectively, and mse_u is the mean squared error of the unrestricted model fit. The first-order model is a restricted form of the mixed-order model and the non-zero asymptote model making this criterion useful for these two comparisons. To assess the relative fit of the mixed-order model and the non-zero asymptote model we compared mean squared errors, which is equivalent to comparing error sums of squares or Akaike information criteria for models with the same number of free parameters.

4. Results

All three models fit among the data reasonably well (Figure 2), though model comparison F statistics favored the non-zero asymptote and mixed-order models over the first-order model in all cases (Table I). Substantial reductions in error sums

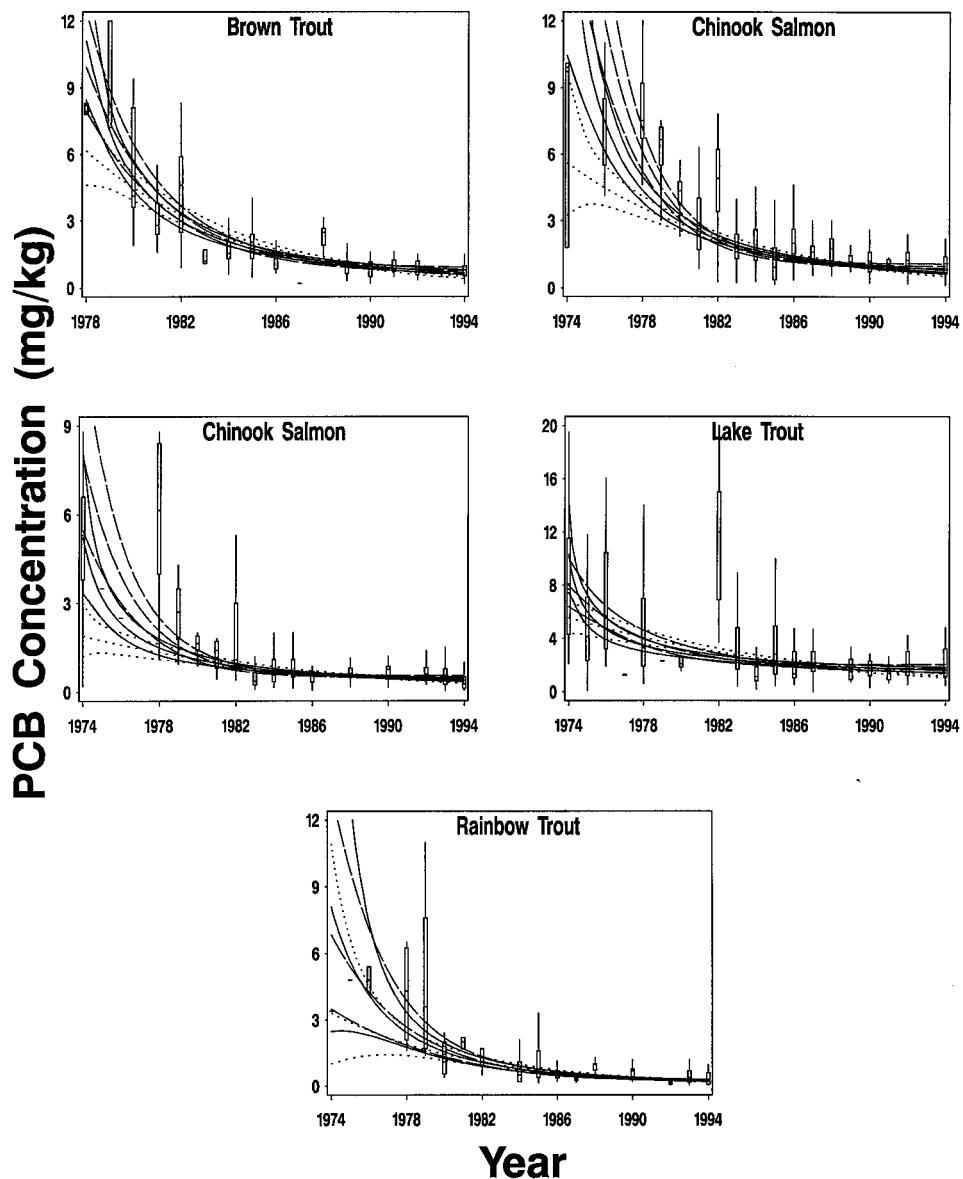


Figure 2. Mixed-order (solid), non-zero asymptote model (coarsely dashed), and first-order (finely dashed) models, ± 2 standard errors, for each of five Lake Michigan fish species. Data are summarized by box and whisker plots with the middle line representing the median, the box representing the inter-quartile range, and the whiskers extending to extreme observations which are no more than 1.5 times the inter-quartile range beyond the quartiles. Models have been fit in the log metric, but for visualization, plotted in the natural metric.

TABLE I

Summary statistics for the three estimated models. Parameter estimates are ± 1 standard error. C_0 for the first-order model is expressed in the log metric

	Brown Trout	Chinook Salmon	Coho Salmon	Lake Trout	Rainbow Trout
n	262	683	466	597	264
First-order model					
C_0	1.82 \pm 0.081	1.73 \pm 0.088	0.64 \pm 0.11	1.71 \pm 0.081	1.20 \pm 0.17
k	0.16 \pm 0.0092	0.11 \pm 0.007	0.087 \pm 0.008	0.078 \pm 0.007	0.14 \pm 0.012
error sum of squares	107.55	475.27	465.04	486.31	245.02
Mixed-order model					
C_0	11.11 \pm 1.51	17.76 \pm 4.72	5.23 \pm 1.16	10.35 \pm 1.70	8.10 \pm 4.80
k	0.10 \pm 0.013	0.082 \pm 0.008	0.12 \pm 0.007	0.010 \pm 0.004	0.17 \pm 0.015
θ	1.69 \pm 0.12	1.68 \pm 0.097	2.00 \pm 0.18	2.95 \pm 0.36	1.39 \pm 0.15
error sum of squares	94.09	439.89	425.05	465.07	240.24
Non-zero asymptote model					
C_0	9.21 \pm 1.08	23.60 \pm 4.23	7.47 \pm 1.50	6.36 \pm 0.87	6.65 \pm 2.27
k	0.32 \pm 0.029	0.38 \pm 0.029	0.39 \pm 0.039	0.25 \pm 0.048	0.25 \pm 0.043
C_a	0.72 \pm 0.085	0.96 \pm 0.054	0.49 \pm 0.030	1.67 \pm 0.18	0.20 \pm 0.044
error sum of squares	94.40	410.59	398.75	466.68	238.72
Model comparisons					
N.-order vs. F.-order	$F_{1,259} = 37.25$	$F_{1,680} = 54.68$	$F_{1,463} = 43.56$	$F_{1,594} = 27.13$	$F_{1,261} = 5.20$
NZA vs. F.-order	$F_{1,259} = 36.14$	$F_{1,680} = 107.08$	$F_{1,463} = 76.99$	$F_{1,594} = 19.64$	$F_{1,261} = 6.89$
N.-order vs. NZA	0.363 vs. 0.364	0.647 vs. 0.604	0.918 vs. 0.861	0.783 vs. 0.786	0.920 vs. 0.915

of squares occurred, with both the mixed-order and non-zero asymptote models, for all species except rainbow trout. Rainbow trout error sums of squares also decreased with the non-zero asymptote and mixed-order models, but the reduction was not as pronounced as among the other species. The first-order model generally predicted low in early and later years, and slightly high in middle years (Figure 2). This pattern was even more apparent when we viewed residuals plots, and results from fitting a straight line (in log space) among data that are curved.

The non-zero asymptote model fit the data best for chinook and coho, while the non-zero asymptote and mixed-order models were about equivalent for brown, lake and rainbow trout, as indicated by the error sums of squares comparison (Table I). Estimated θ s were all above one, ranging from 1.39 for rainbow trout to 2.95 for lake trout. These estimates were all well-determined and reinforce the notion that PCB concentration declines are slowing more rapidly than a first-order model would predict.

While the fit of the non-zero asymptote and mixed-order models are essentially the same for the trout species, the implications of the two models are different. Predicted median PCB concentration declines from 1998–2010, under the mixed-order model, are 18.4, 46, and 57% for lake, brown, and rainbow trout, respectively (Figure 3). For chinook and coho salmon the mixed-order model predicts declines over the same period of 42 and 32%, respectively. The non-zero asymptote model predicts virtually unchanging concentrations for all species.

5. Discussion

Fish total PCB concentration declines are a spatial and temporal integration of many processes, some with large stochastic components, acting on 209 PCB congeners; each congener with distinct chemical properties. The first-order (or pseudo-first-order as it has sometimes been called) model was a useful approach to describe these declines into the 1980s, but recent declines no longer follow the first-order approximation. The mixed-order model is more flexible than the first-order model. The mixed-order model is identical to the first-order model when $\theta = 1$, but is not restricted to a value of one. The mixed-order model can capture a much wider range of behavior. Like the non-zero asymptote model the mixed-order model permits rapid initial declines, followed by very slow secondary declines, but without the imposition of an unyielding asymptotic boundary.

Model uncertainty receives moderate attention, in principle, but minimal attention in practice. When uncertainty is formally addressed, it is usually conditional on a given model structure. However, model selection is also a component of uncertainty, and the choice of model can influence resultant inference. Considering predictions from several models, in concert, and the uncertainty associated with each model, can be helpful for evaluating likely future states. Of the three models we considered, the first-order model predicts the largest future PCB declines

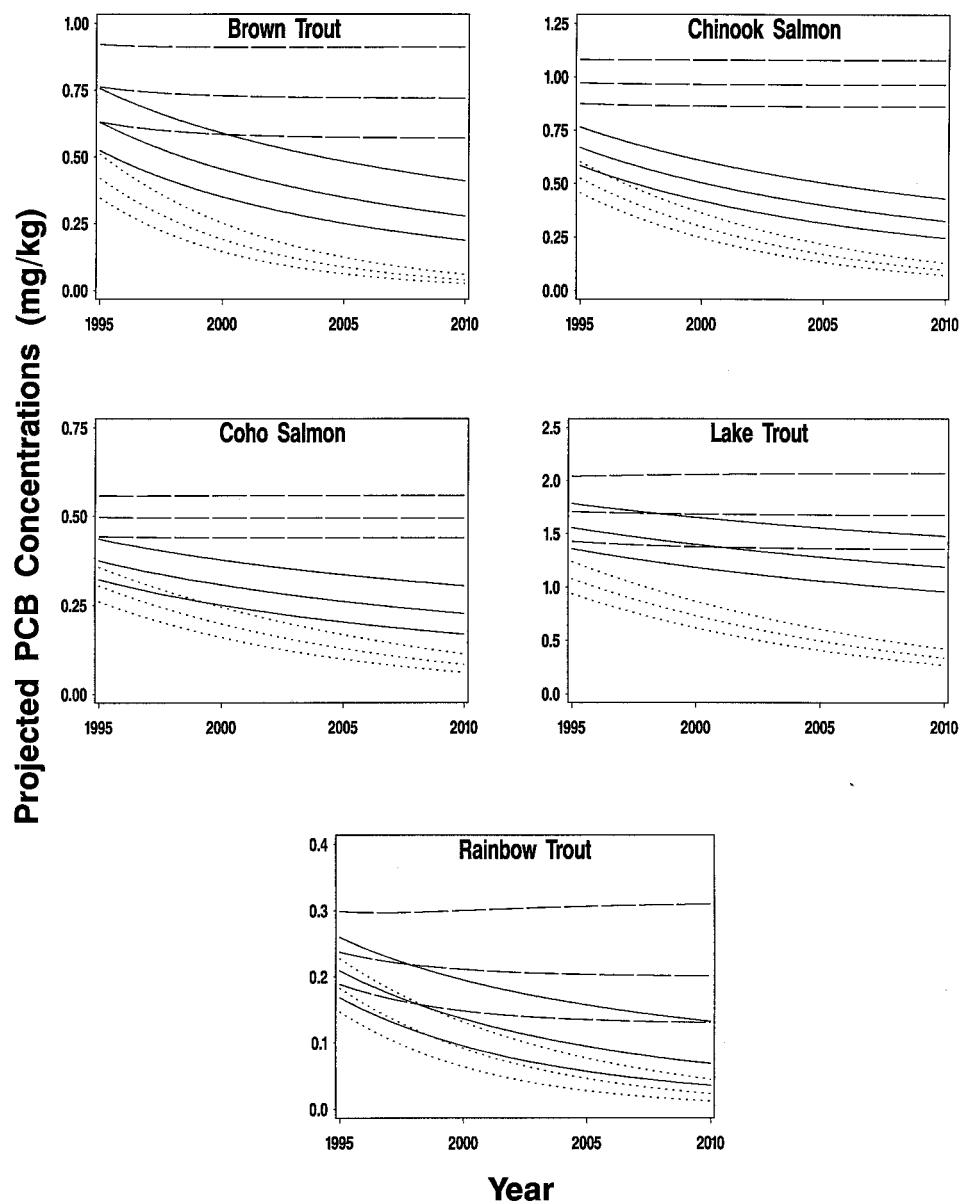


Figure 3. Comparison of predictions from the mixed-order (solid), non-zero asymptote (coarsely dashed) and first-order (finely dashed) models, ± 2 standard errors. Because the models were fitted in the log metric under a normality assumption, predictions expressed in the natural metric depict the estimated median population value.

(Figure 3). However, for all five species the first-order model was the poorest fit to the data. Therefore, we consider these predictions to be relatively unlikely. The non-zero asymptote model predicts essentially no further declines, and for chinook and coho, was the model that fit the data best. Thus, we consider these predictions reasonable for near future planning. Declines predicted by the mixed-order model are intermediate to those of the first-order and non-zero asymptote models, and the mixed-order model and non-zero asymptote models fit the data about equally for brown, lake and rainbow trout. Accordingly, we regard these two models as providing a reasonable range for these species for near future. Similarly, the mixed-order model could be regarded as providing a reasonable lower range for near-future chinook and coho PCB concentrations, constituting a less likely, but plausible, scenario for these two species.

Predictions of the non-zero asymptote and mixed-order models are fairly distinct by about 2002 for brown trout, and coho and chinook salmon, but remain indiscernible for lake and rainbow trout through a forecast horizon of 10–15 yr (Figure 3). For all species except lake trout the mixed-order model predicts relatively high percent declines from 1998–2010. Analysis of 100 fish per year for each species would result in an accumulated sample of 1300 fish, a size that should be sufficient to assess changes over that period, if the mixed-order model predictions are reasonable (Stow *et al.*, 1995). At \$200 per analysis, the total cost is \$100,000 per year, a modest investment relative to the value of the Lake Michigan fishery, particularly if shared among jurisdictions.

Making predictions is somewhat presumptuous. We like to quote our colleague Dr. Arthur Hasler who has been known to wryly observe that ‘Prediction is very difficult, especially when it concerns the future’. However, environmental assessment often necessitates evaluations of likely future conditions. The mixed-order model is another tool to assist in such assessment.

Acknowledgments

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TO NORMALIZE OR NOT TO NORMALIZE? FAT IS THE QUESTION

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Abstract—Concentrations of lipophilic contaminants in biota are frequently corrected for variation in tissue lipid content. Usually, this correction is accomplished by dividing tissue contaminant concentration by lipid concentration to form lipid-normalized data. This ratio-based approach is satisfactory when contaminant concentration varies in direct proportion to lipid content. However, when such a relationship does not exist, erroneous conclusions may be reached. Recent research has emphasized the potential shortcomings of the use of ratio variables. We demonstrate the importance of considering these shortcomings when lipid-normalizing data. Three examples are presented, and an alternative approach based upon the use of analysis of covariance (ANCOVA) is suggested.

Keywords—Lipid normalization Contaminants Organochlorines ANCOVA

INTRODUCTION

Concentrations of lipophilic contaminants are often adjusted for variation in tissue lipid content. This adjustment is performed because it is assumed that lipophilic contaminants accumulate in proportion to tissue lipid content. Lipid-normalized data are used in a variety of applications, including developing biota–sediment accumulation factors [1–6], modeling biomagnification in foodwebs [7,8], and establishing guidelines for lipophilic contaminants in food [9]. The importance of lipid when examining differences in contaminant levels among tissues or species has also been recognized [10,11]. Lipid normalization is usually accomplished by dividing contaminant concentration by lipid concentration to form a ratio (hereafter referred to as the *ratio approach*). It is assumed that this procedure eliminates the influence of lipid covariation.

However, previous studies have found that such ratios only correct for variation in the covariate (in this case, lipid) when the relationship between the two variables is isometric [12–15]. An isometric relationship is one in which the slope of the regression line is constant and the intercept is zero [13]. In reality, isometric relationships may be the exception rather than the rule. Departures from isometry may have unpredictable consequences regarding the interpretation of normalized data created by the ratio approach [13,15,16].

The following examples demonstrate some of these problems and suggest an alternative approach for lipid-normalizing data based upon analysis of covariance (ANOVA). The emphasis in this report is on the adjustment of tissue contaminant concentrations for lipid covariation; however, the ANCOVA approach is equally applicable to other normal-

ization procedures, such as normalizing sediment contaminant concentrations for organic carbon content.

MATERIALS AND METHODS

Three examples are described, two using real data and one using hypothetical data. Statistical comparisons were completed using the general linear model procedure (GLM) in SAS [17]. Comparisons of means were made using Student's *t* test. Linear regressions were used to test for significant relationships between variables.

Example 1

These data were collected by the Canadian Wildlife Service as part of the Great Lakes Herring Gull Surveillance Program [18]. They describe total polychlorinated biphenyl (PCB) concentrations in herring gull (*Larus argentatus*) eggs collected in 1981 from colonies in the Niagara River and Lake Huron (Channel Shelter Island in Saginaw Bay). In this example, differences in total PCB concentrations in herring gull eggs from the two colonies were investigated.

Example 2

Hypothetical data were used in Example 2 to provide an unambiguous interpretation of results and to demonstrate the universality of the techniques presented. Data (mean \pm SD) were generated by hand for contaminant and lipid concentrations, which were representative of those typically found in biomonitoring studies. Mean lipid concentrations and associated variances were within the range of those reported for 12 species of fish (whole body) in the Great Lakes [19–21]. Corresponding contaminant concentrations were generated to form relationships between lipid and contaminant concentrations that were consistent with the assumption that lipophilic contaminants bioaccumulate in proportion to lipid content. This example examined whether there was a signif-

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The opinions expressed in this article are the authors' and are not intended to represent those of Environment Canada.

ificant interspecific difference in hypothetical contaminant concentrations.

Example 3

These data were collected as part of a larger study examining the ecological partitioning of contaminants in forage fish from the St. Clair and Detroit rivers [11]. The data provide a comparison of whole-body hexachlorobenzene (HCB, CAS no. 118-74-1) concentrations in spottail shiner (*Notropis hudsonius*) and bluntnose minnow (*Pimephales notatus*) collected in 1988 from the St. Clair River. This example examined whether there was a significant difference in HCB concentrations between the two fish species.

RESULTS AND DISCUSSION

Example 1

Null hypothesis. There is no significant difference in total PCB concentrations in herring gull eggs collected from the Niagara River and Saginaw Bay in 1981.

Total wet weight PCB concentrations in herring gull eggs from the two colonies are shown in Figure 1. It is evident that there is no difference in mean wet weight PCB concentrations in eggs from the two colonies ($F = 2.24$, $p = 0.151$). There is a significant intercolony difference in lipid concentrations ($F = 48.22$, $p = 0.0001$). However, no statistically significant relationship exists between total PCB concentration and lipid concentration (Saginaw: $r = 0.26$, $p = 0.47$, Niagara: $r = 0.42$, $p = 0.22$). Therefore, the value of lipid normalization must be questioned.

If, however, we had divided PCB concentration by percent lipid to form lipid-normalized (ratio) data, our interpretation would have been different (Fig. 2). Figure 2 shows the lipid-normalized (ratio) PCB concentrations in eggs from the two colonies. The PCB levels are now greater in eggs from

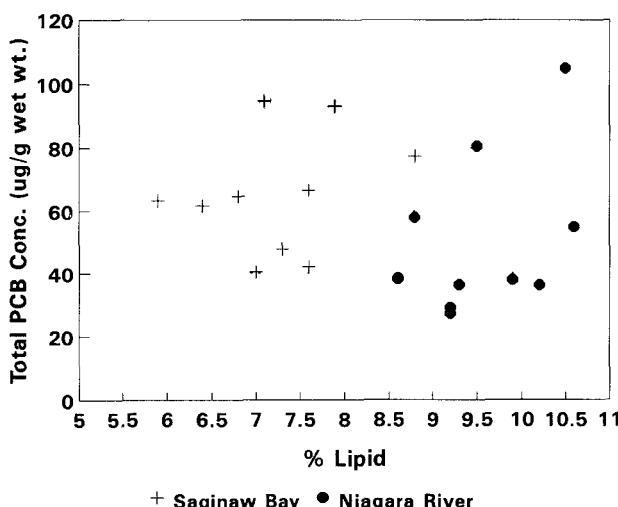


Fig. 1. The relationship between total wet-weight PCB concentrations ($\mu\text{g}/\text{g}$) and percent lipid in herring gull eggs collected in 1981 from Saginaw Bay (crosses) and the Niagara River (circles).

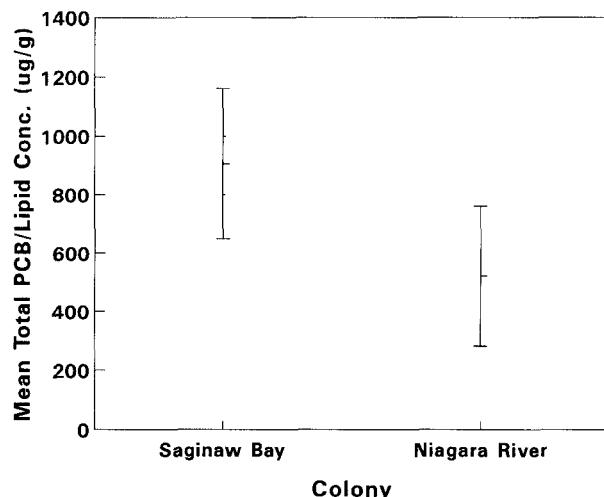


Fig. 2. Mean ($\pm 1 \text{ SD}$) lipid-normalized total PCB concentrations (total PCB/lipid) in herring gull eggs from Saginaw Bay and the Niagara River.

Saginaw Bay than from the Niagara River ($F = 12.02$, $p = 0.0003$). This is inconsistent with our interpretation of the wet-weight data and would have led to the rejection of the null hypothesis. In this example, there was no basis for lipid-normalizing the data, given that there was no statistically significant relationship between lipid and PCB concentration. This example illustrates the potential effect of lipid normalization on the interpretation of contaminant monitoring data. The relationship between contaminant and lipid concentrations should always be examined prior to undertaking any lipid-normalization procedure.

In the Saginaw Bay eggs, there was no obvious relationship between lipid and PCB concentration; hence, we must consider the possibility that by lipid-normalizing the data we introduced more unexplained variability into it. The result, in such cases, is unpredictable, but, as shown in this example, it may radically change our interpretation of the data. In cases where there appears to be no relationship between lipid and contaminant concentration, greater effort should be expended trying to understand the influence of other processes, which may be regulating differences in contaminant bioavailability, before lipid normalization is contemplated. With respect to the Niagara River eggs, it may be argued that there appears to be a relationship between lipid and PCB concentration and, given a sufficiently large sample size, this relationship would be found to be significant. However, even if this were discovered to be true and we did decide to lipid-normalize these data, how should we do it? The following two examples examine this issue.

Example 2: Hypothetical data

Null hypothesis. There is no significant interspecific difference in hypothetical contaminant concentrations.

Hypothetical data were generated for two species, Species A and Species B. Mean ($\pm 1 \text{ SD}$) wet-weight concentrations

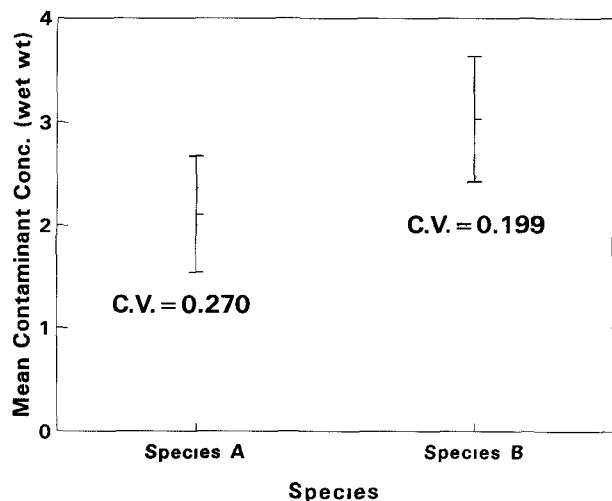


Fig. 3 Mean (± 1 SD) wet-weight hypothetical contaminant concentrations in two species. Coefficients of variation (C.V.) associated with contaminant concentrations are shown under the error bars

are shown in Figure 3. Species B has a higher mean contaminant concentration than does Species A ($F = 12.56, p = 0.002$). Is this difference related to lipid content? When the wet-weight contaminant data are plotted versus percent lipid we see that a positive relationship exists between contaminant and lipid (Species A $r = 0.94, p = 0.0001$, Species B $r = 0.98, p = 0.0001$) (Fig. 4). It is also evident that lipid levels are significantly greater in Species B than in Species A ($F = 36.9, p = 0.0001$). However, when we examine the area where lipid concentrations are similar in the two species we see that Species A has a higher contaminant concentration, and we suspect that this may be the case at all lipid concentrations. There-

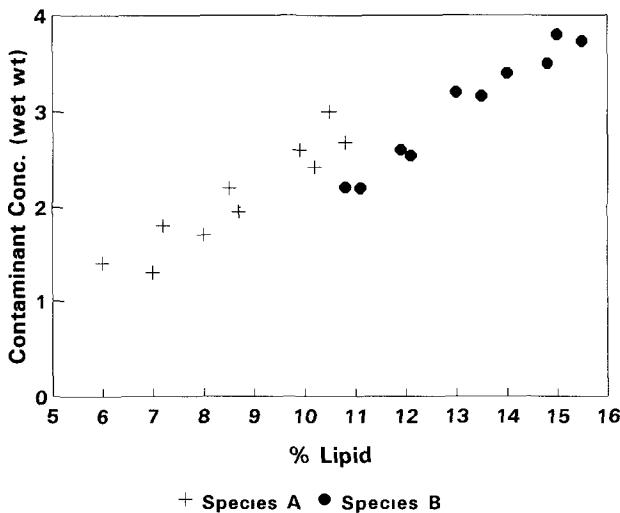


Fig. 4 Relationship between wet weight contaminant concentrations and percent lipid in Species A (crosses) and Species B (circles)

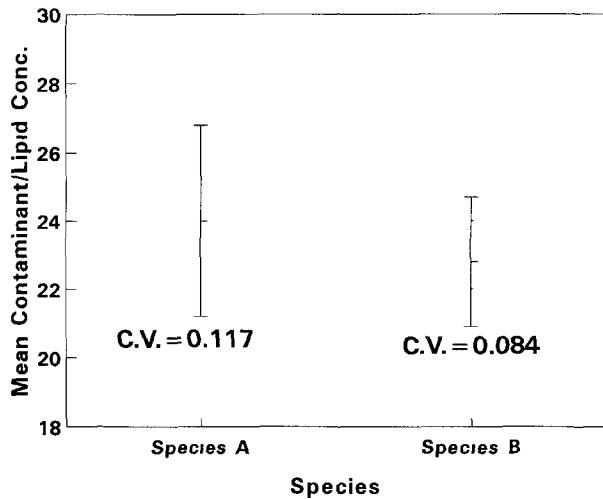


Fig. 5 Mean (± 1 SD) lipid normalized contaminant concentrations (contaminant/lipid) in Species A and B. Coefficients of variation (C.V.) associated with contaminant concentrations are shown under the error bars

fore, we need to remove the confounding influence of lipid on interspecific differences in contaminant concentrations

When the data are lipid-normalized using the ratio approach there are no interspecific differences in mean contaminant concentrations ($F = 1.11, p = 0.31$) (Fig. 5). However, inspection of the wet-weight data indicated that where lipid concentrations were similar in the two species, Species A had higher contaminant levels than did Species B. To explain this apparent inconsistency we need to examine whether the use of the ratio approach has removed the effect of lipid. Figure 6 is a plot of the lipid-normalized (ratio) data versus percent lipid. For both species, there is a positive relationship

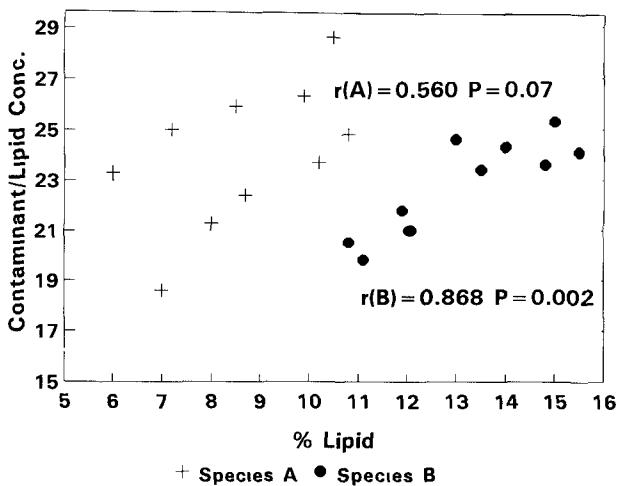


Fig. 6 Relationship between lipid-normalized contaminant concentrations (contaminant/lipid) and percent lipid for Species A (crosses) and Species B (circles). Correlation coefficients (r) and significance level (p) are given for both regressions

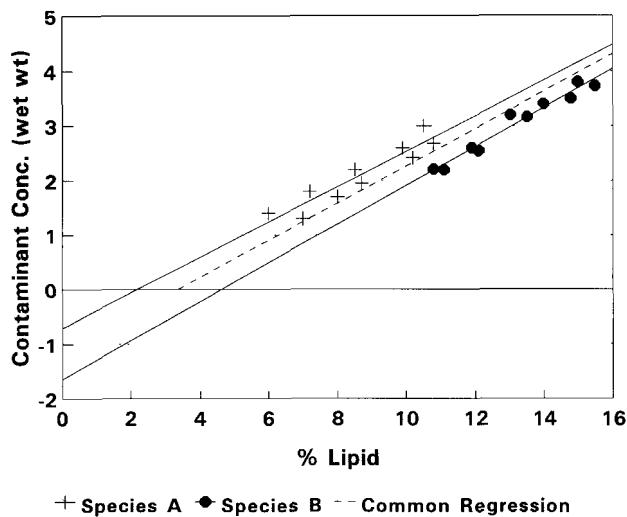


Fig. 7. Relationship between wet-weight contaminant concentration and percent lipid. Species-specific regression lines (solid lines) are shown along with the common regression line for both species (dotted).

between the so-called lipid-normalized (ratio) data and percent lipid (Species A: $r = 0.56$, $p = 0.07$; Species B: $r = 0.87$, $p = 0.002$). The formation of lipid-normalized data using the ratio approach has not removed the effect of lipid.

In this example it is obvious that the ratio approach to lipid normalization was not effective in removing the effect of lipid on contaminant concentration. Another approach to lipid-normalizing data involves the use of analysis of covariance (ANCOVA), which is a simple procedure combining features of regression and ANOVA [22,23].

The initial step in the ANCOVA approach is to use linear regression to examine the relationship between contaminant and lipid concentration. If there is a significant relationship then lipid normalization is appropriate. Where significant departures from linearity occur, the data should

Table 1. Results of ANCOVA for hypothetical data

Source of variation	Type III sum of squares	d.f.	F	p
Species ^a	0.086	1	2.86	0.11
Lipid	5.685	1	189.81	0.0001
Lipid · Species	0.012	1	0.40	0.54
Species ^b	0.587	1	20.30	0.0003
Lipid	5.692	1	197.01	0.0001

Table data tests whether there are significant differences in species contaminant concentrations (Species), whether a significant relationship exists between contaminant concentrations and lipid concentrations (Lipid), and whether there is a significant difference between species in the relationship between contaminant concentration and lipid concentration (Lipid · Species interaction term). Type III sums of squares are adjusted for the covariate.

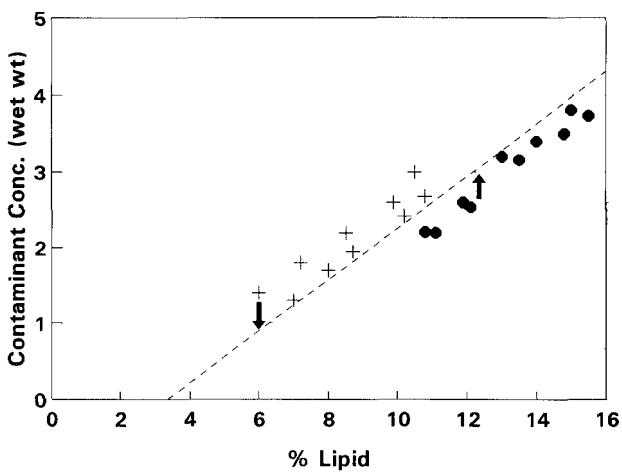
^aResults with interaction term included.

^bResults after interaction term is removed.

be transformed to linearize the relationship between the variables before using a linear model, such as ANCOVA, to analyze the data. The following steps are then performed: (1) Test for significant differences between the slopes of the regression lines for each species (ANCOVA interaction term); (2) If there is no difference, then use the common slope to adjust contaminant concentrations for lipid concentration. If there are significant differences between species regression lines then the individual regression lines can be used to normalize the data [13,15]. In such cases, however, differences between intercepts should be interpreted with caution; and (3). Test for significant interspecific differences between adjusted means (i.e., intercepts).

Figure 7 shows how the ANCOVA approach could be applied to the data in Example 2. ANCOVA indicates that there is no interspecific difference in the slopes of the species regression lines (Table 1, top half). Hence, the interaction term is removed and the ANCOVA is recalculated (Table 1, bottom half), revealing significant lipid and species effects. The common slope (Fig. 7, dotted line) is used to adjust the contaminant concentrations for percent lipid.

In Figure 8, the common regression model is fitted to the data and the residuals are calculated from the regression. These residuals represent the variation in contaminant concentration that remains after the effect of lipid has been removed. Values falling above or below the line have positive or negative residuals, respectively. The residuals are rescaled by adding the grand mean contaminant concentration (in this case 2.57) to each residual. Each measurement has now been adjusted (normalized) for lipid content. When these lipid-normalized data (ANCOVA) are used to examine interspecific differences in contaminant concentrations, Species A has a greater contaminant concentration than does Species



+ Species A ● Species B - Common Regression

Fig. 8. Common regression line for Species A (crosses) and Species B (circles) describing the relationship between wet-weight contaminant concentrations and percent lipid. Residuals are calculated using this common regression. Arrows indicate the formation of residuals, points above the line will have positive residuals, and those below the line will have negative residuals.

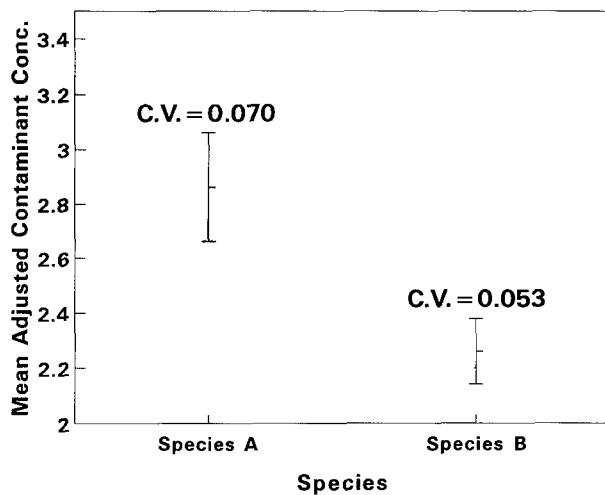


Fig. 9. Mean (± 1 SD) lipid-normalized (ANCOVA) contaminant concentrations in Species A and Species B. Coefficients of variation (C.V.) associated with contaminant concentrations are shown above the error bars.

B ($F = 66.34, p = 0.0001$) (Fig. 9). The null hypothesis is rejected and we conclude that the contaminant concentration in Species A is greater than that in Species B. ANCOVA has removed the variation associated with lipid. Reduction in variation is illustrated in the coefficients of variation (C.V.), which are lower than those for wet weight (Fig. 3) or ratio data (Fig. 5). When these lipid-normalized (ANCOVA) data are plotted versus percent lipid there is no relationship between lipid-normalized contaminant concentration (ANCOVA) and percent lipid (Species A: $r = 0.14, p > 0.5$; Species B: $r = 0.20, p > 0.5$) (Fig. 10). The ANCOVA model has removed the influence of lipid on contaminant concentrations.

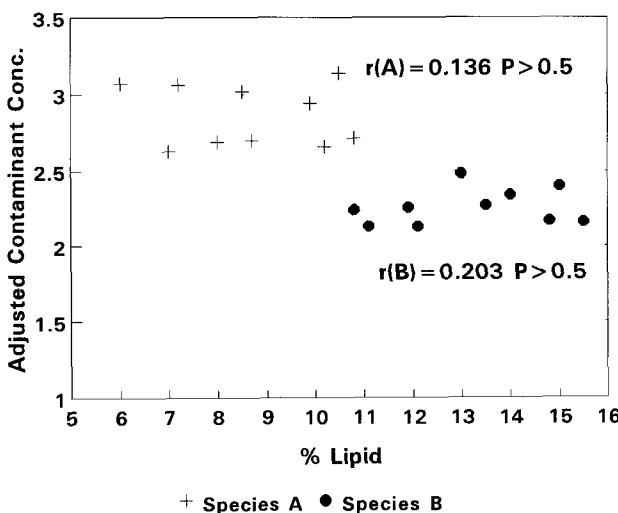


Fig. 10. Relationship between lipid-normalized (ANCOVA) contaminant concentration and percent lipid. Correlation coefficients (r) and significance level (p) are given for both regressions.

Example 3

Null hypothesis. There is no interspecific difference in hexachlorobenzene (HCB) concentrations in two species of forage fish.

Mean wet-weight HCB concentrations are shown for two forage fish species in Figure 11. It is apparent that the bluntnose minnow has higher HCB concentrations than does the spottail shiner ($F = 18.57, p = 0.0001$). Is this difference related to lipid content? In Figure 12, the wet-weight contaminant data are plotted versus percent lipid. There is a positive relationship between HCB and lipid concentration ($r = 0.56, p = 0.0002$). Lipid levels are also marginally greater in the bluntnose minnow ($F = 3.44, p = 0.07$). Therefore, we should correct for interspecific differences in lipid content. When we use the ratio approach to lipid-normalize the data, we find that there is no interspecific difference in lipid-normalized HCB concentration (ratio) ($F = 2.85, p = 0.10$) (Fig. 13). However, the coefficients of variation are greater in the lipid-normalized (ratio) data than in the wet weight data. The precision of the data after lipid normalization (ratio) has been diminished, thereby reducing the power of the statistical test to detect interspecific differences in HCB concentration. Hence, when using the lipid-normalized (ratio) data there is no interspecific difference in HCB concentrations and we accept the null hypothesis.

However, initial inspection of the wet-weight data indicated that there was a significantly greater HCB concentration in the bluntnose minnow. Because the precision of the data has been diminished after lipid normalization, using the ratio approach, we must be skeptical regarding the acceptance of the null hypothesis. Consequently, we want to compare the results discussed above with those obtained when we use the ANCOVA approach to lipid-normalize the data.

When using the ANCOVA approach it is evident that a positive relationship exists between HCB and lipid concen-

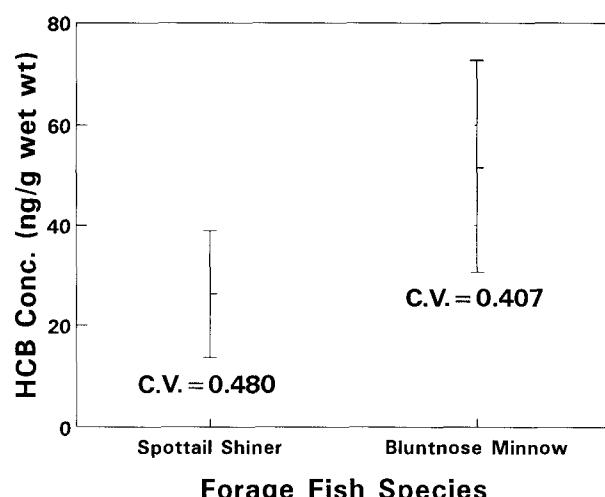


Fig. 11. Mean (± 1 SD) wet-weight hexachlorobenzene concentrations (ng/g) in two species of forage fish, the spottail shiner and bluntnose minnow.

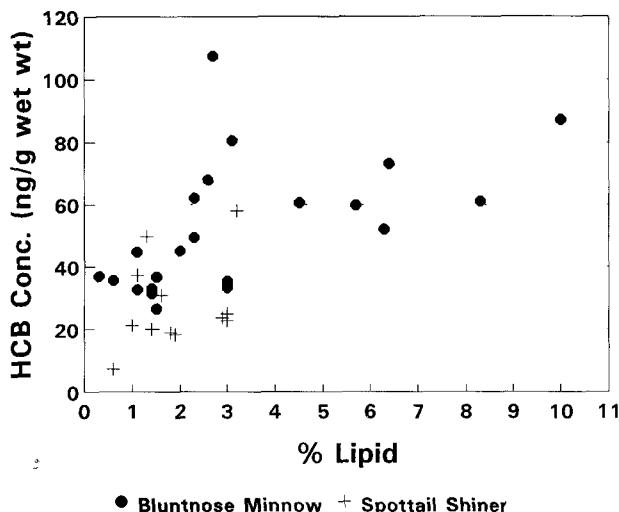


Fig. 12. Relationship between wet-weight hexachlorobenzene concentration and percent lipid for the spottail shiner (crosses) and bluntnose minnow (circles).

trations (Fig. 14). There is no interspecific difference in the slopes of the species regression lines (Table 2, top half). Therefore, the interaction term is removed and the ANCOVA recalculated (Table 2, bottom half), revealing significant lipid and species effects. The common regression line (dotted line in Fig. 14) is used to lipid-adjust the HCB concentrations. When we examine the lipid-normalized HCB (ANCOVA) data it is evident that the bluntnose minnow has significantly greater lipid-normalized HCB concentrations ($F = 15.26$, $p = 0.0001$) (Fig. 15). The coefficients of variation are lower than those resulting from the use of the wet-weight or ratio

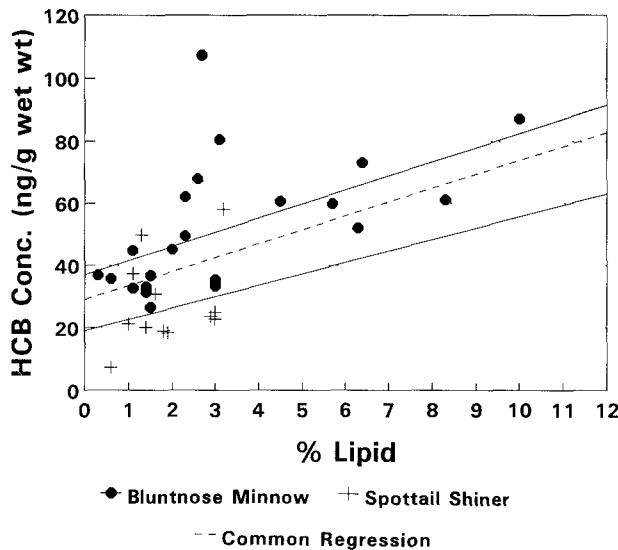


Fig. 14. Relationship between wet-weight hexachlorobenzene concentration and percent lipid. Species-specific regression lines (solid lines) are shown along with the common regression line for both species (dotted line). The common regression line is used to calculate residuals.

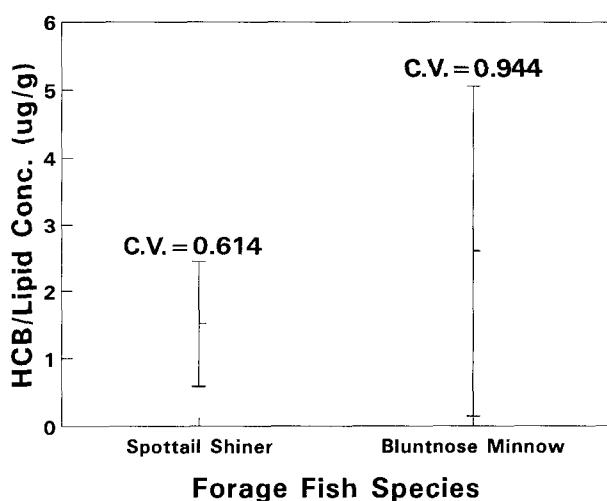


Fig. 13. Mean (± 1 SD) lipid-normalized hexachlorobenzene concentrations (HCB/lipid) in spottail shiner and bluntnose minnow. Coefficients of variation (C.V.) associated with contaminant concentrations are shown above the error bars.

Table 2. Results of ANOVA for forage fish data

Source of variation	Type III sum of squares	d.f.	F	p
Species ^a	606.43	1	2.35	0.13
Lipid	705.83	1	2.74	0.11
Lipid·Species	8.16	1	0.03	0.86
Species ^b	3,407.55	1	13.58	0.0007
Lipid	3,052.87	1	12.16	0.0013

Table data test whether there are significant differences in species contaminant concentrations (Species), whether a significant relationship exists between contaminant concentrations and lipid concentrations (Lipid), and whether there is a significant difference between species in the relationship between contaminant concentration and lipid concentration (Lipid·Species interaction term). Type III sums of squares are adjusted for the covariate.

^aResults with interaction term included.

^bResults after interaction term is removed.

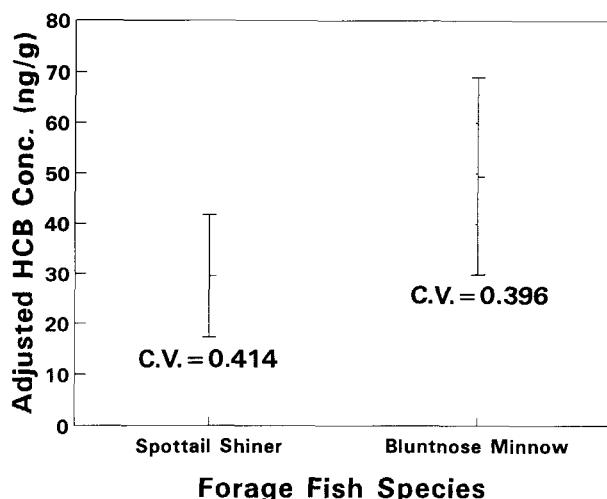


Fig. 15. Mean (± 1 sd) lipid-normalized hexachlorobenzene concentrations (ANCOVA) in spottail shiner and bluntnose minnow. Coefficients of variation (C.V.) associated with contaminant concentrations are shown below the error bars.

ANCOVA approach has identified the fact that factors other than lipid content are important in regulating interspecific differences in HCB accumulation.

CONCLUSIONS

When a significant relationship exists between contaminant and lipid concentrations, then lipid normalization is appropriate. Usually lipid-normalized data are created by dividing contaminant concentration by lipid concentration. This ratio approach may lead to misleading conclusions because of the diminished precision of the data and the reduced power of the statistical test to detect differences. Data may be lipid-normalized using an alternative approach based upon an analysis of covariance (ANCOVA). The ANCOVA approach is easily performed on a microcomputer and takes into consideration the unique properties of individual data sets.

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